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The immune environment in ovarian cancer

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Chapter 1

Introduction to the thesis

01

Epithelial ovarian cancer

Epithelial ovarian cancer (EOC) is the 5th most common cause of cancer death in women in the Western World (1). It represents the most deadly gynecological malignancy with an overall 5 year-survival rate of approximately 40% (2). The poor prognosis is mainly attributable to diagnosis at advanced stage due to a lack of specific early symptoms and screening methods (3). At advanced stage, the disease is characterized by intra-abdominal spread of tumor. Most patients with advanced stage of disease eventually develop therapy-resistant recurrences and succumb to their disease. EOC is a heterogeneous disease, consisting of distinct histological subtypes. The subtypes that can be distinguished are low-grade serous, endometrioid, clear cell, mucinous, and high-grade serous carcinomas (HGSC) and are considered to arise from various primary sites. The most common subtype is HGSC, which accounts for the majority of EOC deaths (3).

Treatment of EOC consists of a combination of cytoreductive surgery and chemotherapy. Patients are treated with a platinum-based chemotherapy regimen, in most cases carboplatin supplemented with paclitaxel. In recent years, it has become evident that the result of surgical cytoreduction is one of the most important clinical variables defining the prognosis of these patients (4), with the group of patients with no visible residual tissue having the best outcome (5). Neo-adjuvant chemotherapy treatment (NACT) has therefore been introduced for patients in whom upfront maximal cytoreductive surgery seems unrealistic. In this approach, patients receive the first 3 cycles of chemotherapy followed by interval cytoreductive surgery. Choice for this treatment option was until recently mostly based on tumor and patient characteristics; patients with large, disseminated tumors and/or with major co-morbidities were eligible for this strategy. NACT is more widely used in recent years, and has led to an increase in the percentage of patients with a completely cytoreduced tumor (6,7). Other recent changes in treatment strategies include strategies targeting angiogenesis using targeted antibodies directed against vascular endothelial growth factor (e.g. Bevacizumab), and poly(ADP-ribose) polymerase (PARP) inhibitors in tumors with alterations in the homologous recombination repair pathway.

Despite these changes in therapy regimens no major improvements in survival rates have been accomplished in the past few decades. Therefore, the search for new strategies offering more success is highly warranted. Increased knowledge in the field of tumor immunology has provided a rationale for the use of immunotherapy in many cancer types, including ovarian cancer.

Tumor immunology

Over a century ago, Dr. William Coley was one of the first to try an immunotherapeutic approach in cancer patients by injecting bacterial toxins in order to activate the immune system (8). This approach was not without success, but due to a lack of explanation of the observed activity, his work was considered controversial.

With the development of other strategies that proved to be successful, such as chemo- and radiotherapy, his approach was quickly forgotten. The idea of involvement of the immune system in tumor control was however not totally rejected, based on the assumption that the immune system should be capable of recognizing the 'non-self' characteristics of the tumor.

Mutations in tumor cells may lead to *de novo* expression of tumor-specific antigens or re- or over-expression of antigens that are expressed only during embryonic development or at low levels in healthy tissues, the so-called tumor-associated antigens. Specific recognition of these tumor antigens by immune cells may induce a response towards tumor cells. Antigen presenting cells (APC) internalize, process, and present tumor antigens that have become available in the tumor environment through tumor cell death. Naïve T cells are primed by these APC in secondary lymphoid structures and subsequent recognition of tumor antigens presented by MHC-I on the tumor surface by cytotoxic T lymphocytes (CTL) may lead to the release of cytolytic granules to eradicate the bound tumor cell. Recognition of tumor antigens presented by MHC-II to CD4+ T cells occurs as well and CD4+/DC/CD8+ cell interactions are necessary for optimal and long-lived effector and memory T cell responses (reviewed in (9)). Furthermore, also CD4+ T cells can show anti-tumor activity.

The past few decades are marked by a renewed interest in the immune involvement in cancer when new methods to precisely study this involvement became available. Localization of specific immune infiltrates in the tumor environment could be studied by use of, amongst others, immunohistochemistry. For EOC, the hallmark study from 2003 showing the prognostic effect of CD3+ tumor-infiltrating lymphocytes (TIL) served as an important starting point. In this study, it was clearly shown that patients with a high influx of CD3+ T cells in the tumor epithelium survive longer (10). Further confirmation of the role of the immune system in tumor control came from studies showing that CD8+ CTL are the most important anti-tumor effector cells, with clear survival differences in patients with a high or low infiltrate of these cells. Further studies on the immune response in these tumors established the suppressive role of other cell types, such as regulatory T cells (Treg), with a high ratio of CD8+ T cells over Treg providing for a longer survival time (11,12).

Tumor microenvironment

In recent years it has become evident that the composition and the distribution of the immune infiltrate in the tumor predict prognosis of patients with various tumor types. In colorectal cancer, this has led to the definition of a local immune signature that better predicts the disease course than traditional staging based on histopathological characteristics (13). Next to the fact that immune surveillance can limit tumor progression directly, the strength of the response may also determine efficacy of conventional therapeutic interventions (14) and in that way contribute to a better prognosis. The immune response is, however, inhibited

by suppressive mechanisms in the tumor microenvironment. Through selection of immune escape variants during a process named cancer immunoediting, tumors manage to escape immune eradication (15,16). Various mechanisms attribute to this shaping of tumor immunogenicity, which is characterized by three successive phases: elimination, equilibrium, and escape. This last escape phase involves multiple factors in the tumor environment, including the downregulation of molecules involved in antigen presentation, the influx of suppressive immune cells types and expression of molecules that inhibit the cytotoxic function of T cells.

The tumor environment comprises multiple cellular subsets that through complex interactions play an essential role in immune evasion and the progression of the disease. By creation of a hostile tumor environment, the ongoing immune response is inhibited. Next to factors in the tumor islands, the stromal compartment of the tumor containing immune cells, fibroblasts, anti-inflammatory cytokines, as well as vasculature and extracellular matrix, also contributes to the suppression. This can either occur through direct inhibition of the tumor-reactive TIL present in the tumor, or through inhibition or even complete circumvention of TIL infiltration. The net result of this is a significant reduction in the anti-tumor activity of the immune system. Next to Treg, the infiltration of the tumor with immunosuppressive cells from the myeloid lineage, such as tumor-associated macrophages (TAM), and myeloid-derived suppressor cells (MDSC) are associated with worse survival (17–19). These cell subsets originate from myeloid precursors in the peripheral blood and undergo specific differentiation and polarization dependent on cues they receive from factors in the tumor environment (20). The conditioning of the tumor microenvironment with anti-inflammatory cytokines (e.g. IL-10 and TGF- β), produced by these suppressive immune subsets as well as by tumor cells themselves, can actively inhibit the TIL response (21). Other important suppressive mechanisms include checkpoint inhibition of activated T cells through CTLA-4 or the PD-1/PD-L1 axis, as well as expression of TIM-3 or LAG3 on T cells. Furthermore, other aspects of the TME that have been associated with active immune suppression in ovarian cancer are, amongst others, expression of indoleamine 2,3-dioxygenase (IDO), FasL, and CD73 (22–24). Knowledge on the exact immune infiltration and immunosuppressive mechanisms in the tumor microenvironment may provide targets for immunotherapy in ovarian cancer.

Current status of immunotherapy

The past few years have been the most successful for immunotherapy thus far, with implementing immunotherapy leading to significant improvements in survival for multiple tumor types. The current successes are mostly the result of strategies releasing checkpoint inhibition in TIL. Immune checkpoints inhibit the activation of T cells following antigen presentation and costimulatory signaling by APCs in order to control the intensity and duration of the immune response. In cancer, tumor cells may express proteins that, by binding to activated TIL, lead to check-

point inhibition in which intracellular mechanisms lead to suppression of these cells. Blockade of CTLA-4, PD-1, or PD-L1 with monoclonal antibodies to prevent activation of checkpoint pathways and to boost the immune response, has led to impressive results in various tumor types. After first successes with targeting CTLA-4 in metastatic melanoma patients (25), prognosis of patients was significantly increased by blockade of either PD-1 or PD-L1 in several solid tumor types (26,27). In ovarian cancer, blockade of PD-L1 in a phase I trial led to an objective response in 1 out of 17 patients (27), and PD-1 blockade in a phase II trial resulted in 2 complete responses out of 20 patients (28), both in heterogeneous patient cohorts.

Other antibody-based therapies are based on targeting the tumor, for example through binding with receptors on the tumor cell, which disrupts important signaling pathways. Another option is antibody-dependent cell-mediated cytotoxicity (ADCC), in which the binding of specific antibodies to tumor cells induces a cellular immune response through retention of a functional Fc domain on the antibody bound to the tumor cell. Examples of monoclonal antibody treatment in ovarian cancer are antibodies targeting vascular endothelial growth factor (VEGF) and HER2. However, the clinical efficacy of such strategies is hampered by the heterogeneity of ovarian tumors. This is reflected by a high variability of target expression on tumor cells and immunosuppressive mechanisms. (29,30).

Other options to boost the immune response towards the tumor include adoptive cell transfer (ACT) by use of tumor TIL or peripheral blood cells that are manipulated to express chimeric antigen receptors (CAR-T). ACT with TIL requires the isolation of these cells from tumor tissue and expansion *ex vivo*, after which TIL are transferred back to the patient. The rationale is that TIL will be enriched for tumor-reactive T cells. ACT was found to be of limited success in ovarian cancer (31) which could be attributable to the fact that in this approach TIL are expanded without selection, and therefore also immunosuppressive Treg and non-reactive T cells will be expanded and infused back into the patient. Another major obstacle for this technique is that it requires a pre-existing immune response, and therefore enough TIL to isolate, while many patients do not have significant infiltrate of tumor-reactive TIL. To overcome this, peripheral autologous T cells can be engineered to recognize tumor targets by expression of a CAR, which is composed of ScFvs specific for a tumor antigen and a T cell signaling domain capable of inducing activation. An important feature of CAR-T is that these cells can be constructed to recognize surface proteins and in that way are not relying on a proper antigen-processing pathway, which is impeded in many tumors, including ovarian cancer (32,33). Challenges of this strategy include lack of persistence and proliferation of the CAR-T *in vivo* due to the immune suppressive environments of tumors, and difficulties in the selection of targets that are expressed on the majority of tumor cells.

Another option to provoke an immune response towards tumors is the use of vaccination strategies. With this strategy, induction of an immune response is achieved through an increase in the presentation of tumor antigens or neoantigens

by antigen-presenting cells, resulting in the generation of tumor-specific CTL. Options include DC vaccines, peptide vaccines, recombinant viral vaccines and vaccination with RNA. So far, success of such strategies has been limited in ovarian cancer, which may in large part be explained by heterogeneity in expression of targeted antigens and the highly suppressive tumor microenvironment (34–36). Therefore, combination strategies of the aforementioned immunotherapeutics with strategies that target the suppressive environment are likely necessary to induce significant immune responses associated with clinical responses in EOC.

Aim of this thesis

In order to improve immunotherapeutic strategies and determine which strategy would be most suitable for a specific tumor type or patient, further knowledge on the immune response in EOC is warranted. This thesis aims to explore the precise nature of the immune response in EOC to find new targets for different immunotherapeutic strategies, both on immune and tumor cells. Data in this regard might give more clues on how to stratify patients for currently applied immunotherapeutic strategies.

Content of this thesis

In **Chapter 2**, we analyzed characteristics of CD20+ T cells in ovarian cancer. *In vitro* experiments showed that co-culturing T and B cells could lead to transfer of HLA-DR and CD20 from the B cell to the T cell, creating CD20+ T cells. This population was analyzed and phenotyped in EOC patient's blood and ascites.

In **Chapter 3** a study is presented in which the expression of interleukin-6 (IL-6), its receptor (IL-6R), and pSTAT3 were studied in order to determine the role of this signaling pathway in ovarian cancer. Furthermore, since IL-6 might attract TAM to the tumor environment, the infiltration of several tumor-infiltrating myeloid cell (TIM) subsets was studied. These factors, together with several well-known factors in these tumor types (e.g. CD8+, CD45RO+, FoxP3+ TIL) were profiled to determine the tumor immune environment of these tumors.

To further differentiate the immune profile and to study the effect of current treatment strategies on this immune profile we created a new homogeneous patient cohort consisting of advanced stage high-grade serous ovarian cancer patients who had all received the same chemotherapy treatment, which is still the preferred current treatment modality in OC patients. The cohort could be split into those patients that had received either surgery (PS) or neo-adjuvant chemotherapy (NACT) as the primary treatment strategy. In **Chapter 4**, these two patient cohorts were analyzed for TIL infiltrate in order to determine whether treatment regimen affects the prognostic value of these cells. Furthermore, the amount of residual tumor tissue after cytoreductive primary or interval surgery was taken into account. To further differentiate with respect to the tumor reactivity of TIL subsets, the phenotype of TIL in these tumors was analyzed. Consequently, the prognostic value

of the less-differentiated CD27+ subset of TIL was determined. Since prognostic benefit from TIL was found differential between patients treated with PS or NACT, differences may exist in the suppressive environment of these tumors. In **Chapter 5** we analyzed the immune infiltrate and PD-L1 expression on tumor epithelium and immune cells in stroma in these two cohorts, in order to determine how these molecules relate to immune cell infiltration and whether these molecules negatively affect the prognostic benefit of TIL.

Strategies to target the tumor to enhance the immune response are also of interest in ovarian cancer. Homogeneously expressed targets are of importance for this purpose. In **Chapter 6** we analyzed the expression pattern of the tight junction protein claudin-6 (CLDN6) on HGSC tumors before and after chemotherapy, since this cell surface protein might serve as a target for therapy. In **Chapter 7** we studied, CLDN18.2, another tight junction protein and potential TAA, in several gynecological malignancies. Next to the targeting potential, tumor-specific expression of this protein also has a possible diagnostic role. Differentiating primary mucinous tumors from metastases of gastrointestinal origin found in the ovary is difficult, though important, since platinum-based chemotherapy for ovarian tumors is not effective for gastrointestinal malignancies. CLDN18.2 was analyzed together with a panel of markers for determination of its diagnostic role for these tumor types.

In **Chapter 8** the implications of these studies for treatment of EOC patients as well as remaining questions for research are discussed and concluding remarks are made.

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